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1) Aoubala, et al., Epitope mapping and immunoactivation of human gastric lipase using five monoclonal antibodies, Eur J Biochem 211 (1-2): 99-104 (1993).

2) Aoubala et al., Immunological technique for the characterization of digestive lipases, Methods Enzymol 286 (Lipases part B), 126-149 (1997).

Thanks a bunch!

Gail Gabel
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Eur. J. Biochem. 211, 99 – 104 (1993)

Epitope mapping and immunoinactivation of human gastric lipase using five monoclonal antibodies

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Five monoclonal antibodies (mAb) directed against human gastric lipase (HGL) have been produced by hybridization of myeloma cells with spleen cells of BALB/c immunized mice. All these mAb belong to the IgG_1 class with a κ light chain. The effects of these mAb on the enzymic activity of HGL were studied and used to define three classes of antibodies, depending upon their immunoinactivation properties. As determined by ELISA and immunoinactivation studies, four overlapping epitopes were found to be part of the functional sites of the enzyme. The mAb appear to be suitable probes for studying the lipid binding and catalytic domains of HGL. The results of the ELISA additivity test were used to describe tentatively the epitopes of HGL in terms of a schematic spatial map.

In humans, the hydrolysis of dietary triacylglycerols begins in the stomach and is catalyzed by one major enzyme; human gastric lipase (HGL) [1 – 3]. HGL is a glycoprotein with a molecular mass of around 50 kDa, which is located in the chief cells of fundic mucosa [4]. *In-vitro* studies have shown that HGL hydrolyses both short-chain and long-chain triacylglycerols at comparable rates [5]. The enzyme is highly stable and active under acidic pH conditions. The optimal pH for HGL activity is 5.4 in the case of long-chain and 6 in the case of short-chain triacylglycerols.

The amino acid sequence of HGL, deduced from the cDNA, consists of 379 residues [6] and shows a conservative pentapeptide (Gly-Xaa-Ser-Xaa-Gly) common to all the known lipases. The essential serine within this pentapeptide is numbered 153 in HGL and 152 in porcine pancreatic lipase [7] as well as in human pancreatic lipase [8]. This serine is known to be part of the Asp-His-Ser catalytic triad to be found in esterases [8, 9].

Chemical modifications in HGL exposed to diethyl *p*-nitrophenyl phosphate and 5,5′-dithiobis(2-nitrobenzoic acid) have shown that the Ser153 together with the single free sulfhydryl group are essential for the hydrolytic activity of HGL [10 – 12]. Using the monolayer film technique, it has been established that neither of these residues is involved in lipid binding [11, 12]. No information about the three dimensional structure of HGL is yet available, although several crystal types have been obtained [13].

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Abbreviations. HGL, human gastric lipase; mAb, monoclonal antibody; *I*, additivity index.

Enzyme. Triacylglycerol lipase (EC 3.1.1.3).

Two functional sites in the lipolytic enzymes (a lipid or interface binding domain and a catalytic domain) have been postulated based on monolayer experiments [14]. In order to further characterize these functional domains of HGL, an immunological approach was adopted here using mAb against HGL as probes for the spatial identification of the domains.

In the present paper, we describe the production and functional characterization of five mAb against HGL.

MATERIALS AND METHODS

Proteins

HGL was purified in the laboratory from human gastric juice, using procedures described previously [15]. The protein had a specific activity of 1000 $U \cdot mg^{-1}$ with tributyrin as substrate and showed a single band upon SDS/gel electrophoresis [16]. The bovine serum albumin and anti-mouse-IgG – peroxidase conjugate used were from Sigma.

Preparation of mAb

Two young female BALB/c mice were immunized with HGL as follows. The first and the second immunizations were carried out with 100 μg HGL emulsified in complete Freund's adjuvant, injected subcutaneously with a 10-day interval. Two weeks after the second injection, the mice were bled and the serum tested in a direct binding ELISA test. The mouse with the highest titre was selected and 100 μg pure antigen were again injected. 10 days later, three fusion-priming intraperitoneal injections of 50 μg HGL, each in 10 mM sodium phosphate, 150 mM NaCl, pH 7.4 (buffer A) were given on three subsequent days. Spleen cells were obtained 1 day after the last injection and fused with non-secreting mouse myeloma

P3X63 Ag8.653 (ATCC) [17, 18]. Cell fusion was performed using poly(ethylene glycol) 1500 (Boehringer Mannheim). The hybridomas in hypoxantin/aminopterin/thymidin medium (medium A) were seeded into six microtiter tissue-culture plates containing murine peritoneal macrophages as a feeder layer. 10 days after the fusion, 180 µl culture supernatant were removed from each well in order to test the anti-HGL activity in a direct-binding ELISA test.

Direct-binding ELISA test for screening anti-HGL antibodies

When not stated otherwise, the following buffers were used for all ELISA procedures. Coating buffer, buffer A; wash medium, buffer A containing bovine serum albumin (5 g/l) and Tween 20 (0.5 g/l); saturating buffer, buffer A containing bovine serum albumin (5 g/l); substrate solution, orthophenylene diamine (Sigma) (0.4 g/l) in 0.05 M sodium phosphate/citrate, pH 5, containing fresh hydrogen peroxide (0.4%); stop solution, 2 M sulfuric acid.

The screening of hybridoma supernatants was carried out by performing solid-phase immuno assays using 96-well microtiter poly(vinyl chloride) plates (Maxisorb, Nunc). The plates were coated with 500 ng HGL/well in coating buffer overnight at + 4°C and washed. The free sites were saturated with saturating buffer for 2 h at room temperature. Hybridoma supernatants (50 µl) were added to each well and incubated for 1 h at room temperature. MAb-producing hybridomas were detected with peroxidase-conjugated anti-mouse IgG antibody (Sigma). The substrate solution of peroxidase was used to quantify the positive clones. The reaction was stopped with sulfuric acid and the absorbance (A) was read on an automatic plate reader (Dinatech) at 492 nm. Between each step in the assay, the plates were rinsed three times with wash medium.

Isotype identification

A mouse monoclonal antibodies isotyping kit (Amersham) was used to determine the antibody class of the five anti-HGl mAb. The class-specific were set up according to the manufacturers instructions, with culture supernatants of each clone after limiting dilutions.

Purification of mAb

Positive hybridomas were cloned using the limiting dilution technique. For ascites production, 2.5×10^6 hybridoma cells were injected intraperitoneally into BALB/c mice. mAb were purified from mouse ascitic fluids by precipitation with 50% saturated ammonium sulfate followed by affinity chromatography on Protein-A – Sepharose CL-4B (Pharmacia). Fractions containing antibodies were dialyzed against buffer A containing 0.02% sodium azide, concentrated to 4 mg/ml using Centriprep 30 concentrators (Amicon) and passed through 0.22-µm Millipore filters. The purities of the IgG preparations were checked by SDS/polyacrylamide gel electrophoresis.

Direct binding ELISA test on purified mAb

For the binding studies on mAb, assays were carried out with the ELISA test as described above with minor modifications. Microtiter plates were coated with 2.5, 5 or 20 ng pure HGL in 50 µl coating buffer. After washing and saturation of the free sites with a bovine serum albumin solution, the plates were incubated for 1 h at room temperature with 50 µl mAb solution containing 0.125 – 2.0 µg antibodies.

The epitope specificity of the mAb was studied using the ELISA additivity method described by Friguet et al. [19]. mAb were co-titrated in pairs. mAb 4-3, 25-4 and 35-2 gave low absorbance values with 2.5 ng HGL/well (see Fig. 1). In order to co-titrate these antibodies more efficiently, the ELISA additivity test was also carried out with 5 ng HGL/well. In the experiment with 83-15 and 218-13, the plates were coated with 2 ng HGL/well.

mAb affinity constants for HGL

The measurement of the dissociation constants of each mAb-HGL complex was carried out with an ELISA test as described by Friguet et al. [20]. Briefly, various concentrations of HGL were first incubated in solution with a constant concentration of each mAb until equilibrium was reached. The concentration of free antibody was then determined by performing a direct ELISA test as described above.

Enzymic activity assays

To test the influence of each mAb on the lipolytic activity of HGL, we used three types of substrates differing in their fatty acid chain length. Assays on tributyrin (Fluka) and soybean oil (commercial grade) emulsified in gum arabic were carried out with the bulk pH-stat method under standard conditions as described by Gargouri et al. [5]. The kinetics of the hydrolysis of 1,2-didecanoyl-*sn*-glycerol films by HGL [21] were recorded, with or without incubation of the enzyme with each mAb, using the barostat technique previously described by Verger and de Haas [22].

Tributyrin and soybean oil assays

A fixed amount of HGL (7.3 µg and 30 µg in the case of tributyrin and soybean oil, respectively) was incubated (20 µl final volume) with each mAb at various molar ratios in buffer A for 1 h at 37°C.

Monolayer assay

HGL (0.15 nM final concentration) was incubated with each mAb (75 pM final concentration) in buffer A for 1 h at 37°C). The residual activity of HGL was determined by adding each mixture to the lipolysis systems described above. Control experiments were carried out with no antibody.

Protein measurement

The concentrations of solutions of purified HGL and mAb were determined spectrophotometrically at 280 nm using absorption coefficients of $A^{1\%}_{1cm} = 15.8$ and 14, respectively.

RESULTS

Production of mAb

Hybridomas prepared by fusing spleen cells with myeloma cells were placed in 576 wells containing the selective medium (medium A) and mouse peritoneal macrophages as feeder cells. 10 days after the fusion, 422 wells produced one or more clones of which 52 clones had anti-HGL activity in a direct binding ELISA test and five of them (4-3, 25-4, 35-2, 83-

Table 1. Dissociation constants of mAb-HGL complexes and immunoinactivation of HGL by mAb. The residual activity measured with three substrates corresponds to the lipase activity remaining after 1 h of incubation of HGL with each mAb at a fixed molar ratio (mAb/HGL = 0.5). In the case of 1,2-didecanoyl-*sn*-glycerol, the residual activity was measured at 35 mN · m^{-1} [21].

MAb numbering	K_d	Residual activity of HGL		
		tributyrin	soybean oil	1,2-didecanoyl-*sn*-glycerol (35 mN · m^{-1})
	10^7 M	%		
4-3	1.03	39	8	19
25-4	1.58	38	25.5	26
35-2	0.19	40	17.5	27
83-15	0.02	88	54	42
218-13	8.80	92	95	90

15 and 218-13) were selected and cloned using the limiting-dilution technique. The five clones selected were then cultured for storage and production of antibodies in mice. mAb were then purified from ascitic fluid as described in Materials and Methods. The concentration of mAb obtained from the ascitic fluid (15 – 36 ml) varied over 2 – 7 mg/ml. Using an isotyping kit, all five mAb were found to be of the IgG_1 isotype with a κ light chain. The values of the dissociation constants determined with each HGL-mAb complex in solution, using the ELISA procedure as described by Friguet et al. [20], are given in Table 1.

Epitope specificity of mAb

To test whether the mAb recognized different epitopes on HGL, the ELISA double-antibody-binding test (ELISA additivity test) developed by Friguet et al. [19] was used. The enzyme was first titrated by each mAb by performing a direct ELISA test. The titration curves given in Fig. 1 show that the five mAb showed different binding properties with HGL adsorbed on a polyvinyl chloride plate. The maximal signal obtained with increasing mAb concentrations occurred in all cases at 10 µg/ml (500 ng/well), which corresponds to the saturation of all the accessible epitopes. Fig. 1 also indicates that the binding of antibodies to adsorbed HGL increased with the amount of antigen present on the plate. Competition between antibodies for the antigen was expressed by means of additivity indexes [19]. This index (expressed as a percentage) makes it possible to evaluate the simultaneous binding of two monoclonal antibodies to the antigen. The five anti-HGL mAb were studied in all possible pairs and the additivity index values are given in Table 2.

Effects of mAb on the catalytic activity of HGL

Three experimental protocols (methods A, B and C) have been developed at our laboratory for studying the inactivation of a lipase on emulsified systems [23, 24]. Basically, one can either incubate the enzyme with the inactivator (method A) or inject the inactivator during the enzyme action (method B). Both methods gave qualitatively similar results with the mAb 4-3 and 25-4 (data not shown). The effects of each mAb incubated with the enzyme (method A) on the lipolytic activity of HGL were studied using the following three substrates: tributyrin, soybean oil and 1,2-didecanoyl-*sn*-glycerol.

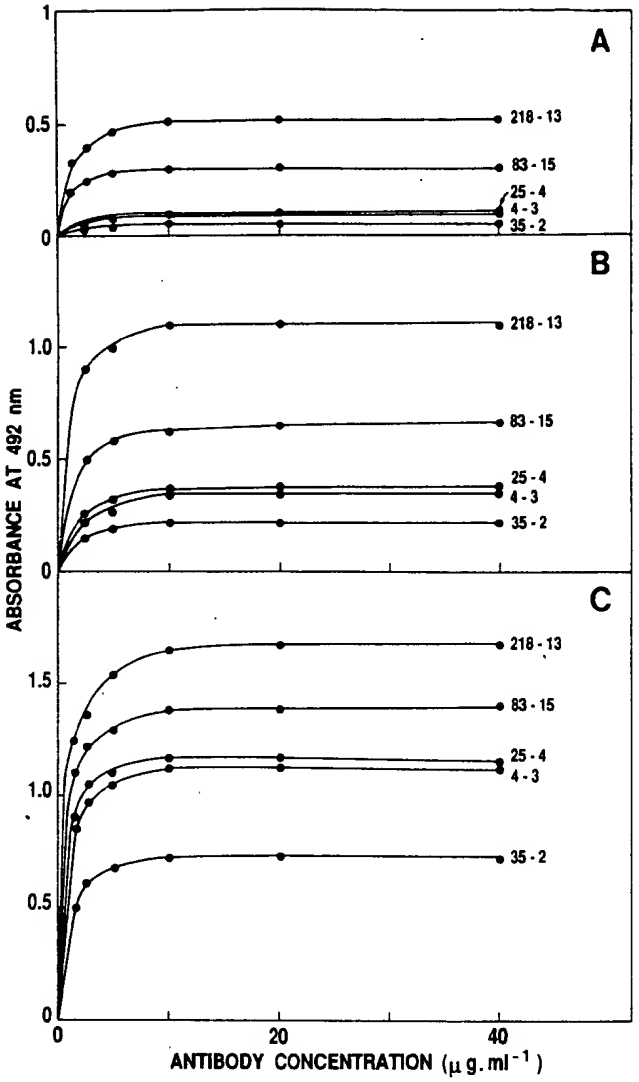

Fig. 1. Titration curves of the five anti-HGL mAb with ELISA test. The plates were coated with 2.5 (A), 5 (B) or 20 ng (C) HGL/well.

HGL activity on tributyrin and soybean oil (Fig. 2)

Three mAb classes could be defined, class 1 included mAb 4-3, 25-4 and 35-2 (Fig. 2A), class 2 included mAb 83-15 (Fig. 2B) and class 3 contained mAb 218-13 (Fig. 2C). The three mAb of class 1 yielded very similar inhibition patterns, reaching a maximum at a mAb/HGL molar ratio of 0.5. Fig. 2A illustrates typical mAb 35-2 inhibition curves with tributyrin and soybean oil as HGL substrates. In the case of class 2, which contained mAb 83-15 (Fig. 2B), no significant effects on the HGL lipolytic activity were observed when the substrate was tributyrin, whereas on soybean oil the HGL activity was inhibited by 50%. With class 3 (Fig. 2C), mAb 218-13 had no inhibitory effect on the HGL activity with either tributyrin or soybean oil. It is worth noting that the inhibition curves did not form a plateau (Fig. 2A and B). At a constant HGL concentration, the residual activity decreased with increasing mAb concentrations, reaching a maximum at a mAb/HGL molar ratio of 0.5. Higher molar ratios led to a relative increase in the HGL residual activity.

Table 2. Additivity indexes (I, expressed as percentages, above the line).

According to Friguet et al. [19], $I = 100\left(\frac{2A_{1+2}}{A_1 + A_2} - 1\right)$ where A_{1+2} is the absorbance obtained in the ELISA test with the mixture of two mAb at a 1:1 molar ratio while A_1 and A_2 are the absorbances obtained with each mAb, respectively. In each square, the two values represent the results of two separate experiments.

Epitopic distance (in nm, below the line). In each square the upper values, for each pair of epitopes, are the distances calculated from the average of the above I values. The lower values are the exact distances measured on the model constructed from the spatial map of the centers of the epitopes given in Fig. 4. In order to calculate the distances (d) between the centers of two overlapping epitopes from the experimentally determined I, we assumed that the I are inversely related to the overlapping surfaces between pairs of epitopes which have a circular shape with a radius of $r = 1.5$ nm [29] and we used the following equation:

$$\frac{I}{100} = 1 - \left(\frac{2}{\pi}\operatorname{arc\,tg}\frac{2}{d}\sqrt{r^2 - \frac{d^2}{4}} - \frac{d}{\pi r^2}\sqrt{r^2 - \frac{d^2}{4}}\right).$$

mAb numbering	4-3	25-4	35-2	83-15	218-13
4-3		25.4 16.4	47.0 38.3	46.5 38.9	46.3 42.0
25-4	0.5 0.5		25.4 31.4	20.5 31.1	43.7 43.7
35-2	1.0 1.0	0.7 0.7		51.8 55.8	54.7 57.4
83-15	1.0 1.0	0.6 0.7	1.3 1.2		75.0 n.d.
218-13	1.1 1.1	1.1 1.1	1.4 1.4	1.9 1.9	

*HGL activity on 1,2-didecanoyl-*sn*-glycerol monolayers (Fig. 3)*

In order to optimize the inhibitory effect of mAb against HGL, the enzyme was incubated for 1 h at 37°C with each mAb at a constant mAb/HGL molar ratio of 0.5. We checked that the optimal inhibition of HGL activity tested on monolayers was obtained at mAb/HGL molar ratios of 0.5 as previously observed in the case of the bulk assay systems (data not shown). The lipolytic activity of HGL was measured at various surface pressures ($10-35$ mN $\cdot$ m^{-1}). As shown in Fig. 3, HGL does not significantly hydrolyse 1,2-didecanoyl-*sn*-glycerol films at surface pressures below 15 mN $\cdot$ m^{-1}. Above this value, the activity increases rapidly, reaching a maximum value at 35 mN $\cdot$ m^{-1}. When HGL incubated with each of the five mAb was tested, the above classification of the mAb, based on bulk assay systems, was confirmed.

DISCUSSION

Sarda and his group [25] have prepared mAb against porcine colipase in order to probe the surface regions of colipase that are essential for activity. Colipase is a non-enzymic protein (10 kDa) which makes it possible for pancreatic lipase to bind to its substrate in the presence of bile salt and prevents surface denaturation of the enzyme. Using Fab fragments derived from mAb against porcine colipase, these authors observed that the loss of biological properties of the protein stemmed from specific interactions between the antibodies

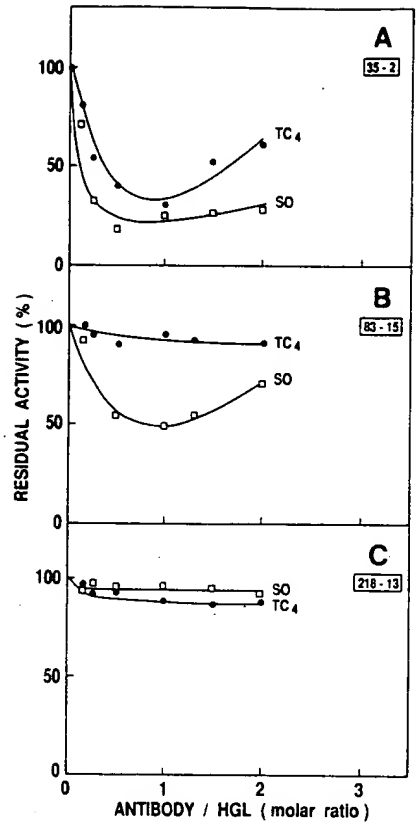

Fig. 2. Effects of mAb on HGL activity. A fixed amount of HGL, 7.3 µg (●) or 30 µg (□), was incubated (20 µl final volume) with each mAb at various molar ratios in buffer A. After 1 h of incubation at 37°C, the relative residual enzymic activity of HGL was measured on either tributyrin (TC_4; ●) or soybean oil (SO; □) (A) mAb 35-2; (B) mAb 83-15; (C) mAb 218-13.

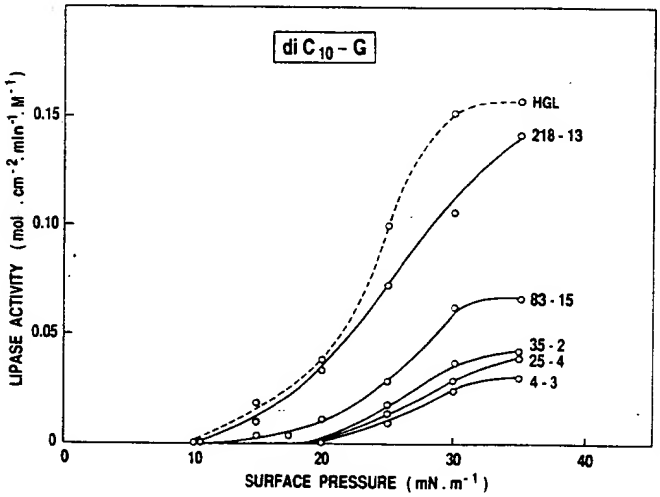

Fig. 3. Effects of the five mAb on HGL activity determined using 1,2-didecanoyl-*sn*-glycerol films (di C_{10}-G) as substrate at various surface pressures. HGL (0.15 nM final concentration) was incubated with each mAb (75 pM final concentration). After 1 h of incubation at 37°C, each mixture was injected into the reaction compartment of a 'zero-order' trough (volume 120 ml, surface 103.9 cm^2) containing 100 mM glycine/HCl, pH 5.4, 100 mM NaCl, 10 mM $CaCl_2$.

with the lipid-binding site and not with the lipase-binding domain of colipase [26].

We previously observed that the lipolytic activity of HGL was inhibited by specific polyclonal antibodies (data not shown). It is not possible, however, to study precisely how polyclonal antibodies inactivate the enzyme. mAb have the advantage of reacting with well-defined epitopes on the antigen. We therefore produced and characterized five mAb against HGL in order to use them as topological probes on the native enzyme.

With regard to the immunoreactivity of HGL, the results of the ELISA test showed that the five anti-HGL mAb gave signals of various amplitudes when reacting with HGL adsorbed on polyvinyl chloride plates (Fig. 1). Among other possible factors, this finding shows the influence of the orientation of HGL adsorbed on an hydrophobic surface. Unlike the inhibitory mAb (4-3, 25-4 and 35-2), the non-inhibitory mAb 218-13 reacted maximally in a direct binding ELISA test with adsorbed HGL. Some of the hyrophobic epitopes of HGL involved in the adsorption of the enzyme to the lipid/water interface may have been less accessible in a direct ELISA test. Consequently, the mAb specific to hydrophobic regions could be in a less favorable position during the screening procedure. It is obviously easier to use a direct ELISA test than, e.g. a radioimmunoassay to select mAb from hybridoma cultures. In the case of proteins with functional hydrophobic regions such as lipolytic enzymes, an indirect ELISA test involving specific polyclonal antibodies adsorbed as the first layer on polyvinyl chloride plates should theoretically yield randomly orientated antigenic regions.

We found that only some mAb had an inhibitory effect on the lipolytic activity of HGL (Table 1). These mAb may be directed towards epitopes which either include residues involved in functional sites (lipid binding or catalytic sites) or are very nearby, thus preventing access by the substrates to the catalytic site. In the case of mAb 4-3, 25-4 and 35-2 complexed to HGL, both the short-chain and long-chain substrates were not hydrolyzed, indicating that the epitopes included the active site. Maximal HGL inactivation by mAb 83-15 was observed only when medium-chain and long-chain acylglycerols were used (see Figs 2 and 3). Possibly, in this case, the epitope was located near the catalytic site, giving steric interference, by mAb 83-15 fixation. Smaller substrates such as tributyrin may, however, have easier access to the catalytic site. It may therefore be possible to interpret the inactivating properties of mAb 83-15 in terms of a partial steric hindrance.

With the inhibitory mAb, an increase in the residual HGL activity was observed at mAb/HGL molar ratios higher than 0.5 (see Fig. 2). Assuming HGL possesses a single non-repeating epitope for these inhibitory mAb, a decrease of mAb/HGL affinity with an excess of mAb was to be expected, due to the negative cooperativity between the two antibody-combining sites [27].

In order to elucidate the organisation of structural domains within the hexokinase molecule, Wilson and Smith [28] used peptide-mapping techniques in conjunction with immunoblotting methods to build up a three-dimensional structure of the epitopes. For the same purpose, we adopted the quantitative ELISA additivity test developed by Friguet et al. [19]. The values obtained range over 20.5–75% (Table 2). From these results, it emerged that four (4-3, 25-4, 35-2 and 83-15) out of the five anti-HGL mAb recognize closely overlapping epitopes in the same antigenic region. Based on the results of the ELISA additivity test, we have tentatively described the epitopes of HGL by means of a schematic spatial map (Fig. 4). The additivity indexes (I) between mAb (see Table 2) have been taken to be inversely related to the overlapping surfaces between pairs of epitopes. For instance, a 100% I means no overlap between epitopes, conversely a total overlap is characterized by a 0% I. If one assumes first that an epitope has a relatively flat surface (7 nm^2) with protuberances and depressions formed by the amino acid side chains of the combining antibody site [29] and secondly that the complex formation between a mAb and its antigen does not induce any conformational changes that might affect the subsequent binding of other mAb [29], according to the formula given in the legend of Table 2, it is possible to calculate the distances between the centers of the five epitopes and to build a model (Fig. 4) which closely fits the calculated epitopic distances (see Table 2). A general overview of the resulting model compared on scale with the assumed dimensions of HGL, confirms that the various epitopes are partially overlapping and are all located in the same antigenic region of HGL. Furthermore, each corner of the polyhedron (Fig. 4) corresponds in fact to the center of gravity of each epitope, since a typical epitope comprises around 16 amino acid side chain residues, as in the case of the three-dimensional structure of the Fab/lysozyme complex [29].

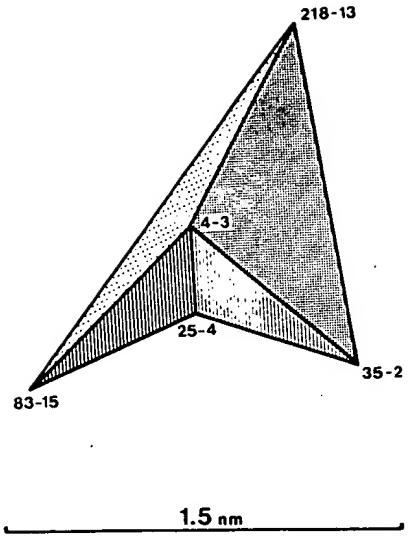

Fig. 4. Schematic map of the centers of the epitopes recognized by the five anti-HGL mAb (4-3, 25-4, 35-2, 83-15 and 218-13). This model was based on the data given in Table 2. A 1.5-nm scale is represented indicating that epitopes, having an average radius of 1.5 nm, are partially overlapping and are located in the same antigenic region of HGL (assumed dimensions 4.8 × 11.3 × 9.1 nm).

In conclusion, four of the five anti-HGL mAb recognize overlapping epitopes belonging to the same antigenic region of the enzyme. Immunoinactivation studies show that this region is essential for the expression of HGL enzymic activity. The four mAb directed towards HGL functional sites provide useful tools for future studies on the interfacial binding site involved in the adsorption of the lipase to the lipid/water interface.

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